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REMARKS/ARGUMENTS

Claims 49, 50, 59-72 and 77-88 are pending after the entry of this amendment. As a result of this amendment, claims 42-48, 53, 54, 58 and 73-76 are canceled without prejudice to renewal of the canceled subject matter in a related patent application.

Claims 49, 50 and 71 were amended to incorporate the limitations of canceled claim 58 or 73-76. Claims 59, 63, and 85 were amended to correct dependency or improve clarity. No new matter is added by this amendment. Because the claim amendments incorporated limitations of previously pending now canceled claims their entry under 37 C.F.R. §1.116 is believed to be proper and is requested. It is believed that these amendments place the claims in condition for allowance and/or for better position for appeal, and the Examiner is respectfully requested to enter the amendment.

I. Objections to the Specification

Applicants were requested to replace the priority application at page 1, line 3. Accordingly, the specification is amended above to correctly state priority information.

II. Claim Objections

Claims 47 and 48 were objected to various informalities. This objection is rendered moot by cancellation of the claims.

III. Rejections Under 35 U.S.C. § 103(a)

Claims 42-52 and 55-88 were rejected as obvious over Pack et al. WO 96/37621 and further in view of Davis et al. U.S. Patent No. 6,265,564 and Desnick et al., U.S. Patent No. 5,580,757. Claims 42-48, 58, and 75-76 are cancelled. This rejection is respectfully traversed as applied and as is might be applied to the presently pending claims.

The Examiner has correctly recognized that none of the references by themselves teach the claimed invention. However, the rejection argues that the combination of the references would be obvious and that this combination at least taught the invention as claimed within the previously pending claims.

It is applicants' position that the combination of the references is not obvious particularly in that the primary reference to Pack et al. teaches away from the use of immunoglobulin-derived multimerizing domains shown at page 6, line 8 to page 8, line 5 of WO 96/37621. In view of the amendments made to the independent claims, applicants further argue that the use of the specific multimerizing components makes the combination of the references even more unobvious. However, notwithstanding applicants' position that the references do not provide a *prima facie* case of obviousness, it is applicants' position that the presently pending claims overcome any *prima facie* case of obviousness by demonstrating unexpected results. A more detailed discussion of the cases cited within the rejection, the art cited, specific distinguishing features between that art and the claimed invention such is put forth below.

The analysis under §103(a) requires that each cited prior art reference be assessed "as a whole" to determine (1) its scope and content, (2) the difference between the cited prior art reference and the claimed invention, and (3) the level of ordinary skill in the art at the time the invention was made. Graham v. John Deere Co., 383 U.S. 1, 148 USPQ 459 (1966). A rejection of a claim for obviousness over a combination of prior art references must establish that (1) the combination produces the claimed invention and (2) the prior art contains a suggestion or motivation to combine the references in such a way as to achieve the claimed invention. In re Vaeck, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). A *prima facie* case of obviousness is potentially refutable with evidence of unexpected results or other objective indicia of nonobviousness. In re Soni, 54 F.3d 746, 34 USPQ2d 1684 (Fed. Cir. 1995).

The invention as claimed. Amended claim 49 is drawn to an isolated nucleic acid molecule encoding a fusion polypeptide comprising a first subunit comprising at least one copy of the receptor binding domain of angiopoietin-1, the first subunit being fused to the N-terminal end of a multimerizing component, and a multimerizing component being fused at its C-terminal end to a second subunit comprising at least one copy of the receptor binding domain of angiopoietin-1, wherein the multimerizing component is an immunoglobulin derived domain selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG. Amended

claim 50 is similar except drawn to a fusion polypeptide having the receptor binding domain of Ang-2 as a first and second subunit.

Claims 59-60 is drawn to a fusion polypeptide encoded by the isolated nucleic acid molecule of claim 49 or 50; claim 60 specifies that the fusion polypeptide is multimerized. Claim 61 is drawn to a composition comprising the multimerized fusion polypeptide of claim 60; claim 62 defines the multimer as a dimer. Claims 63-69 are vectors and host-vector systems, and claim 70 is drawn to a method of producing the fusion polypeptide.

Claim 71 is drawn to an isolated nucleic acid molecule encoding a fusion polypeptide comprising tandem receptor binding domains and a multimerizing component wherein the multimerizing component is an immunoglobulin derived domain selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG and the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2. Claim 77 is drawn to a fusion polypeptide encoded by the isolated nucleic acid molecule of claim 71; claim 78 specifies that the fusion polypeptide is multimerized. Claim 79 is drawn to a composition comprising the multimerized fusion polypeptide of claim 78; claim 80 defines the multimer as a dimer. Claims 81-87 are vectors and host-vector systems, and claim 88 is drawn to a method of producing the fusion polypeptide.

WO 96/37621 as a whole. Pack et al. describe multimeric polypeptides with two or more functional domains capable of self-multimerization. The invention is achieved with small peptidic multimerization devices, e.g., domain1-linker1-multimerization device-linker2-device2 (page 10, lines 13-14). Their peptidic multimerization device is 30-110 amino acids and capable of self-assembly. Functional domains may be from a general list, including domains that bind a defined target substance, catalyze a reaction, inhibit an enzyme, bind or block a receptor binding site, or bind a metal ion (page 22, lines 3-9). Experimental results with functional domains of antibody fragments show that tetramerization of bifunctional fusion proteins increased avidity (Example 1). Pack et al. specifically teach away from use of immunoglobulin-derived multimerizing domains (see page 6, line 8 to page 8, line 5).

US 6,265,564 as a whole. Davis et al. teach fusion proteins that contain Ang-1-Fc and modified and chimeric Ang-1-Fc constructs capable of activating the TIE-2 receptor. It also describes an Ang-1 deletion variant that binds but does not activate the TIE-2 receptor (col. 10, lines 27-33); similarly an Ang-1 variant in which the fibrinogen-binding domain of Ang-1 is replaced with that of Ang-2 (col. 10, lines 40-45).

US 5,580,757 as a whole. Desnick et al. describes α-galactosidase – protein A fusion proteins.

The analysis under 35 USC § 103(a). The following analysis discusses the legal basis for finding that the Examiner has not established a *prima facie* case of obviousness:

1. Pack et al. is not combinable with Davis et al. because Pack et al. teach away from making the claimed invention.

Pack et al. teach against the use of immunoglobulin-derived multimerizing components because of glycosylations problems, expression problems caused by the enormous size of the CH2, CH3, CH4 domains, and folding efficiency caused by size. The invention of Pack et al. is presented as advantageous because of the features "of a relatively small size, low immunogenicity, and high yields of functional material" (page 8, lines 25-29) partially on the basis that the Pack et al. multimers do not contain an immunoglobulin –derived component.

The Federal Court of Appeals has specifically instructed that references cannot be combined when they "diverge from and teach away from the invention at hand" (In re Fine, 837 F.2d 1071, 5 USPQ2D 1596) (Fed. Cir. 1988); W. L. Gore & Assoc. v Garlock, Inc., 721 F.2d 1540, 1550, 220USPQ 303, 311) (Fed. Cir. 1983). Accordingly, Applicants submit that it is inconsistent with current patent case law to combine Pack et al.'s teaching against use of an Fc component with Davis et al.'s Ang1/2-Fc constructs.

2. Pack et al. do not teach forming a multimer with two or more functional domains from a protein that already exists in nature as two or more functional domains.

The problem addressed by Pack et al. is to enhance weak monovalent forces by multiplying the number of interactions (page 2, lines 15-17) to increase binding strength (page 2, lines 28-30). Multivalent interactions are taught as useful for achieving a synergistic gain in binding strength (page 2, lines 28-29) for functional domains that are found as single domains. Park et al. specifically distinguish their invention of the addition of a second functional domain from the teachings of the prior art (page 8, line 25 to page 9, line 4) ("A further limitation of WO 92/03569 is that it does not teach the preparation of multimers having two or more distinct functional domains per fusion protein") (page 8, lines 18-20).

Pack et al. is directed to enhancing binding of a functional domain to its target by increasing the number of interactions through multivalency. The functional domains described are generally a laundry list of "many different types of functional domains" which "may bind to a defined target substance, or catalyze reaction of a defined substance, or inhibit the action of an enzyme, or bind or block a receptor binding site, or bind to a metal ion" (page 22, lines 3-9) including antibody fragments and combinations of "unrelated functional domains such as enzymes, toxins, cytokines, kinases, phosphatases, lectins, peptide hormones, cell adhesion proteins including integrins, metal-binding domains, peptidic vaccines, bioactive peptides, or soluble cell surface proteins such as the CD molecules of leucocytes or parts thereof" (page 9, lines 10-21). The functional domains are characterized as domains that normally exist as a single component.

By contrast, angiopoietin-1 (ang-1) exists in nature as a modular structure consisting of a receptor binding domain, a dimerization motif, and a superclustering motif that forms variable-sized multimers. The fully assembled molecule is a hexamer consisting of two heterotrimeric building blocks ("the C domains of the fibrinogen α, β and γ chains form a coiled-coil structure, resulting in a heterotrimer. Two of these these heterotrimers are linked via interchain disulfide bonds among the cysteines contained within the N-terminal domains") (Davis et al. (2003) Nature Structural Biology 10:38-44) (copy enclosed).

Accordingly, Applicants submit that Pack et al. do not render the instant invention obvious because it does not teach the generation of a molecule having two or more functional domains for a molecule that already exists in nature with two or more functional domains.

3. Pack et al. do not teach a fusion protein having the same functional domains at each end.

Park et al. specifically distinguish their invention of the addition of a second functional domain from the teachings of the prior art (page 8, line 25 to page 9, line 4) ("A further limitation of WO 92/03569 is that it does not teach the preparation of multimers having two or more distinct functional domains per fusion protein") (page 8, lines 18-20).

4. Davis, et al. neither teaches nor suggests the activity of multimerized ligands in accordance with the present invention.

Davis, et al. describes the binding of Ang-1 fibrinogen domain-Fc dimers as well as anti-Fc antibody clustered preparations. However, one skilled in the art could not predict that such fibrinogen domains made as single chain dimers that self-multimerize would mimic the activity of the native Ang-1 and Ang-2 ligands; i.e. act as agonists or antagonists, respectively.

5. The engineered Ang1-Fc-Ang1 tetramer has a different functionality.

Angiopoietin-1 (Ang-1) acts via the endothelial receptor tyrosine kinase TIE2. As shown in Davis et al. (2003) *supra*, the ang-1 fibrinogen binding domain (F_1) (Fig. 1b, Davis et al. (2003) *supra*) does not activate TIE2 receptor in endothelial cells, nor does it activate endothelial TIE2 receptor as a dimer when fused to an Fc domain (F_1-Fc). However, the tetravalent molecule formed from multimerization of two F_1-Fc-F_1 molecules exhibits agonist activity. Accordingly, the engineered tetramer converts F_1 from an antagonist to an agonist.

While Pack et al. envisions enhancing the functionality of a molecule by increasing valency, nothing in Pack et al. teaches one of skill in the art to change an antagonist to an agonist.

6. The addition of Davis et al or Desnick et al. do not achieve the claimed invention.

Applicants respectfully submit that the combination of Pack et al. with either Davis et al. or Desnick et al. is irrelevant to the patentability of the instant claims as neither Davis et al. or Desnick

et al. compensate for the Pack et al. instructions not to use an immunoglobulin-derived multimerizing component.

Conclusion

For the above reasons, Applicants submit that the Examiner has failed to establish a *prima facie* case of obviousness as required under the analysis required under § 103(a). In light of the above amendments and arguments, Applicants contend that all claims are now in condition for allowance, and respectfully request such action.

Fees

No fee is deemed necessary in connection with filing this Amendment. However, if any fee is necessary, authorization is hereby given to charge the amount of any such additional fee to Deposit Account No. 18-0650.

Respectfully submitted

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Includes Text of Claims being Canceled in this Amendment

Claims 1–41. Canceled

Claim 42. **(canceled)** An isolated nucleic acid molecule encoding a fusion polypeptide wherein the fusion polypeptide comprises a first subunit comprising at least one copy of a receptor binding domain of a ligand, the first subunit being fused to the N-terminal end of a multimerizing component, and the multimerizing component being fused at its C-terminal end to a second subunit comprising at least one copy of a receptor binding domain of a ligand, wherein the multimerizing component is an immunoglobulin-derived domain.

Claim 43. **(canceled)** The isolated nucleic acid molecule of claim 42, wherein the receptor binding domains of the first and second subunits are the receptor binding domain from the same ligand.

Claim 44. **(canceled)** The isolated nucleic acid molecule of claim 42, wherein the receptor binding domain of the first subunit is a receptor binding domain derived from a different ligand than the receptor binding domain of the second subunit.

Claim 45. **(canceled)** The isolated nucleic acid molecule of claim 43, wherein the receptor binding domain of the first and second subunit is the fibrinogen domain of angiopoietin-1.

Claim 46. **(canceled)** The isolated nucleic acid molecule of claim 43, wherein the receptor binding domain of the first and second subunit is the fibrinogen domain of angiopoietin-2.

Claim 47. **(canceled)** The isolated nucleic acid molecule of claim 44, wherein the receptor binding domain of the first subunit is the fibrinogen domain ~~is~~ of angiopoietin-1 and the receptor binding domain of the second subunit is the fibrinogen domain of angiopoietin-2.

Claim 48. **(canceled)** The isolated nucleic acid molecule of claim 44, wherein the receptor binding domain of the first subunit is the fibrinogen domain of angiopoietin-2 and the receptor binding domain of the second subunit is the fibrinogen domain of angiopoietin-1.

Claim 49. (currently amended) An isolated nucleic acid molecule encoding a fusion polypeptide wherein the fusion polypeptide comprises a first subunit comprising at least one copy of the receptor binding domain of angiopoietin-1, the first subunit being fused to the N-terminal end of a multimerizing component, and the multimerizing component being fused at its C-terminal end to a second subunit comprising at least one copy of the receptor binding domain of angiopoietin-1, wherein the multimerizing component is an immunoglobulin derived domain selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Claim 50. (currently amended) An isolated nucleic acid molecule encoding a fusion polypeptide wherein the fusion polypeptide comprises a first subunit comprising at least one copy of a receptor binding domain of angiopoietin-2, the first subunit being fused to the N-terminal end of a multimerizing component, and the multimerizing component being fused at its C-terminal end to a second subunit comprising at least one copy of the receptor binding domain of angiopoietin-2, wherein the multimerizing component is an immunoglobulin derived domain selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Claims 51-52. (Canceled)

Claim 53. (Withdrawn) The isolated nucleic acid molecule of claim 43, wherein the ligand is selected from the group consisting of the EPH family of ligands.

Claim 54. (Withdrawn) The isolated nucleic acid molecule of claim 44, wherein the ligands are selected from the group consisting of the EPH family of ligands.

Claims 55-57. (Canceled)

Claim 58. **(canceled)** The isolated nucleic acid molecule of claim 42, wherein the immunoglobulin derived domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Claim 59. (currently amended) A fusion polypeptide encoded by the isolated nucleic acid molecule of claim 49 or 50 ~~claims 42, 43, or 44~~.

Claim 60. (previously presented) The fusion polypeptide of claim 59, wherein the fusion polypeptide is multimerized.

Claim 61. (Previously presented) A composition comprising the multimerized fusion polypeptide of claim 60.

Claim 62. (Previously presented) The composition of claim 61, wherein the multimer is a dimer.

Claim 63. (currently amended) A vector which comprises the isolated nucleic acid molecule of claim 49 or 50 ~~claims 42, 43, or 44~~.

Claim 64. (currently amended) An expression vector comprising an isolated nucleic acid molecule of ~~claims 42, 43, or 44~~ claim 49 or 50, wherein the nucleic acid molecule is operatively linked to an expression control sequence.

Claim 65. (Previously presented) A host-vector system for the production of a fusion polypeptide which comprises the expression vector of claim 64, in a suitable host cell.

Claim 66. (Previously presented) The host-vector system of claim 65, wherein the suitable host cell is a bacterial cell, yeast cell, insect cell or mammalian cell.

Claim 67. (Previously presented) The host-vector system of claim 66, wherein the suitable host cell is *E. coli.*

Claim 68. (Previously presented) The host-vector system of claim 66, wherein the suitable host cell is a COS cell.

Claim 69. (Previously presented) The host-vector system of claim 66, wherein the suitable host cell is a CHO cell.

Claim 70. (Previously presented) A method of producing a fusion polypeptide which comprises growing cells of the host-vector system of claim 66, under conditions permitting production of the fusion polypeptide and recovering the polypeptide so produced.

Claim 71. (currently amended) An isolated nucleic acid molecule encoding a fusion polypeptide, wherein the fusion polypeptide comprises more than one copy of a receptor binding domain of a ligand, each copy fused in tandem, and wherein either the N-terminal or the C-terminal ends of the tandem receptor binding domains is fused to a multimerizing component, wherein the multimerizing component is an immunoglobulin-derived domain selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Claim 72. (previously amended) The isolated nucleic acid molecule of claim 71, wherein the receptor binding domains are fused contiguously.

Claim 73. (previously amended) The isolated nucleic acid molecule of claim 71 or 72, wherein the ligand is not a member of the EPH family of ligands.

Claim 74. (previously amended) The isolated nucleic acid molecule of claim 71 or 72, wherein the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2.

Claim 75. (**canceled**) The isolated nucleic acid molecule of claim 71 or 72, wherein the multimerizing component comprises an immunoglobulin derived domain.

Claim 76. (**canceled**) The isolated nucleic acid molecule of claim 71, wherein the immunoglobulin derived domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Claim 77. (previously presented) A fusion polypeptide encoded by the isolated nucleic acid molecule of ~~claims~~ 71.

Claim 78. (previously presented) The fusion polypeptide of claim 77, wherein the fusion polypeptide is multimerized.

Claim 79. (previously presented) A composition comprising the multimerized fusion polypeptide of claim 78.

Claim 80. (previously presented) The composition of claim 79, wherein the multimerized fusion polypeptide is a dimer.

Claim 81. (previously presented) A vector which comprises the isolated nucleic acid molecule of claim 71.

Claim 82. (previously presented) An expression vector comprising a nucleic acid molecule of claim 71, wherein the nucleic acid molecule is operatively linked to an expression control sequence.

Claim 83. (previously presented) A host-vector system for the production of a fusion polypeptide which comprises the expression vector of claim 82, in a suitable host cell.

Claim 84. (previously presented) The host-vector system of claim 83, wherein the suitable host cell is a bacterial cell, yeast cell, insect cell or mammalian cell.

Claim 85. (currently amended) The host-vector system of claim 84, wherein the suitable host cell is ~~E. coli~~ *E. coli*.

Claim 86. (previously presented) The host-vector system of claim 84, wherein the suitable host cell is a COS cell.

Claim 87. (previously presented) The host-vector system of claim 84, wherein the suitable host cell is a CHO cell.

Claim 88. (previously presented) A method of producing a fusion polypeptide which comprises growing cells of the host-vector system of claim 83, under conditions permitting production of the fusion polypeptide and recovering the polypeptide so produced.

Angiopoietins have distinct modular domains essential for receptor binding, dimerization and superclustering

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Angiopoietins are a recently discovered family of angiogenic factors that interact with the endothelial receptor tyrosine kinase Tie2, either as agonists (angiopoietin-1) or as context-dependent agonists/antagonists (angiopoietin-2). Here we show that angiopoietin-1 has a modular structure unlike any previously characterized growth factor. This modular structure consists of a receptor-binding domain, a dimerization motif and a superclustering motif that forms variable-sized multimers. Genetic engineering of precise multimers of the receptor-binding domain of angiopoietin-1, using surrogate multimerization motifs, reveals that tetramers are the minimal size required for activating endothelial Tie2 receptors. In contrast, engineered dimers can antagonize endothelial Tie2 receptors. Surprisingly, angiopoietin-2 has a modular structure and multimerization state similar to that of angiopoietin-1, and its antagonist activity seems to be a subtle property encoded in its receptor-binding domain.

Angiopoietins are a family of vascular growth factors that collaborate with members of the vascular endothelial growth factor family to regulate vascular and lymphatic vessel growth[1–6], acting *via* the endothelial receptor tyrosine kinase Tie2 (ref. 7). Although angiopoietin-1 (Ang1) seems to be an obligate activator of the Tie2 receptor, angiopoietin-2 (Ang2) seems to have context-specific effects, activating this receptor on some cells while blocking Tie2 activation on other cells or under different conditions[3,6,8,9]. Most receptor tyrosine kinases are activated by ligand-induced dimerization[10,11], often by ligands that are themselves dimeric; however, some growth factors mediate more elaborate multimerizations[12,13]. We[1] and others[14] have found that the angiopoietins are produced as oligomers, but nothing is known about the precise features or the functional significance of this oligomerization. In an attempt to understand the manner in which Ang1 and Ang2 differentially regulate the Tie2 receptor, we decided to further define the multimeric nature of these related growth factors.

N-C-F structure of Ang1

The amino acid sequence of Ang1 can be divided into three regions with distinct features (Fig. 1*a*). The sequence begins with a stretch of ~50 residues that bear little resemblance to other known proteins (denoted 'N' for N-terminal region) and that we anticipated might constitute a separate functional domain. Following this is a domain of ~215 amino acids that displays features characteristic of proteins known to assume a coiled-coil conformation (denoted 'C' for coiled-coil domain). The C-terminal domain of Ang1 consists of ~215 amino acids and is homologous to the C-terminal domain of fibrinogen (denoted 'F' for fibrinogen-like domain). The overall structural features of Ang1 (Fig. 1*a*) are reminiscent of those found in fibrinogen, which also has the N-C-F form[15]. In fibrinogen, the fully assembled molecule is a hexamer consisting of two heterotrimeric building blocks. That is, the C domains of the fibrinogen α, β and γ chains form a coiled-coil structure, resulting in a heterotrimer. Two of these heterotrimers are then linked *via* interchain disulfide bonds among the cysteines contained within the N-terminal domains of the respective subunits to form hexamers.

Functions of C and F domains

On the basis of structural analogies to fibrinogen, we proposed that the N and C domains of the angiopoietins might primarily serve to multimerize the F domain, and the F domain might primarily have the function of receptor binding. To test this hypothesis, we produced and characterized the isolated F domain of angiopoietin-1 (denoted Ang-F_1; Fig. 1*b*). Light scattering analysis[16], which provides an estimate of the mass of a protein under native conditions in solution, confirmed that Ang-F_1 was monomeric (Fig. 2*a*). In contrast to a construct containing just the N and C domains (denoted Ang-N_1C_1; Fig. 1*b*), which was never observed to bind to Tie2 (data not shown), Ang-F_1 could indeed bind to Tie2 (Fig. 2*b*). However, Ang-F_1 bound to Tie2 less strongly than native Ang1 (Fig. 2*b*), which is consistent with the possibility that native Ang1 consisted of higher order multimers of F and bound Tie2 more tightly because of avidity effects. As would be expected for a reagent that bound to receptors but could not dimerize them, Ang-F_1 could

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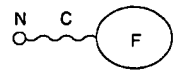
a



b

Variant (F-domains)	Schematic diagram	Binding IC_{50} (nM)	Activation Fibr.	Activation Endo.
Ang-N_1C_1 (0)	N C	no binding	ND	ND
Ang-F_1 (1)	F	435	–	–
Ang-C_1F_1 (2)		94	+	–
Ang1 (4,6,8,...)	+higher order	1.5	+	+
clustered Ang-C_1F_1 (4+)		ND	+	+
Ang-F_1-Fc (2)		71	+	–
clustered Ang-F_1-Fc (4+)		ND	+	+
Ang-F_1-F_1-Fc (4)		ND	+	+
Ang-F_1-Fc-F_1 (4)		9.7	+	+

not activate either endogenous Tie2 receptors expressed in endothelial cells (Fig. 3*a*, lane 3) or ectopically expressed Tie2 receptors introduced into fibroblasts (Fig. 3*c*, lane 2). At high concentrations, Ang-F_1 actually inhibited Angl-mediated activation (Fig. 3*b*, lane 3).

The construct Ang-C_1F_1, consisting of the C and F domains of Angl, was found to be a homodimer by light scattering (Fig. 2*a*), indicating that the coiled-coil region of Ang-C_1F_1 forms a double-stranded structure (Fig. 1*b*), in contrast to the coiled-coil region of fibrinogens, which form heterotrimers. Consistent with this finding, mass spectroscopic analysis of fragments proteolytically generated from native Angl demonstrated that the single cysteine found within the C domain (residue 245 of the native Angl protein) is involved in an intermolecular disulfide bridge between two coiled-coil domains — that is, a tryptic fragment was observed with a mass of 4,196 ± 1 Da, corresponding to a dimer of the tryptic peptide QQLELMDTVHNLVNLC$_{245}$TK disulfide-linked *via* the Cys245 residues.

Fig. 1 Structure and activity of Angl and Angl variants. ***a***, A schematic diagram of Angl domain architecture. ***b***, Schematic diagrams of presumed structures and multimerization states of Angl variants and their ability to bind Tie2, as well as their ability to activate Tie2 receptors in fibroblasts and endothelial cells. Numbers in parentheses refer to the F domain multimericity of each variant. The IC_{50} values refer to the experiment shown in Fig. 2*b*. Other results are referred to in the text. 'ND' is 'not determined'.

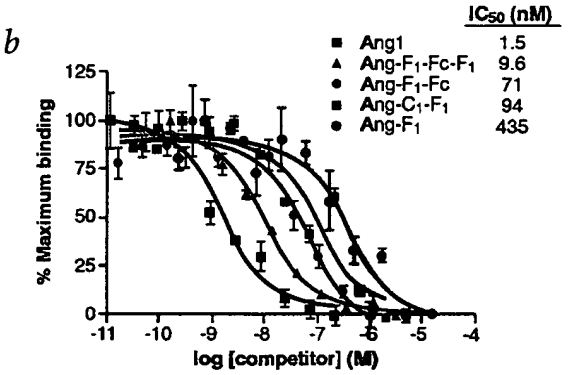
Requirement for superclustered ligand

As might be expected, binding studies revealed that Ang-C_1F_1 dimers bound Tie2 more strongly (about five-fold lower IC_{50} in competition experiments) than the monomeric Ang-F_1 (Fig. 2*b*), presumably because of avidity effects. However, despite being dimeric and binding with a higher apparent affinity, Ang-C_1F_1 could not activate Tie2 receptors on endothelial cells (Fig. 3*a*, lane 4). Instead, saturating concentrations of Ang-C_1F_1 actually inhibited endothelial Tie2 activation by native

a

Protein	M_W (kDa) of monomer	M_W (kDa) by light scattering	F-domain multimericity
Ang-F_1	25.0	24.8	1.0
Ang-C_1F_1	49.7	95.0	1.9
Ang-F_1-Fc	51.0	103.7	2.0
AngF_1-Fc-F_1	76.4	147.5	3.8
AngF_2-Fc-F_2	76.1	159.6	4.2

b



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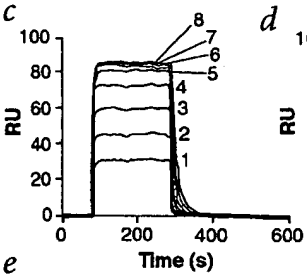

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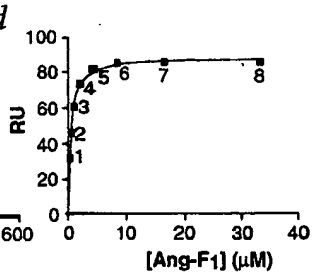

e

Capturing antibody	Captured Tie2-Fc	Ligand bound at saturation				Ang/Tie2 molar ratio
	fmol	Ligand	M_W	RU	fmol	
anti-Fc	8.1	Ang-F_1	27.2	209	7.7	0.95
anti-Fc	8.1	Ang-F_2	27.4	198	7.2	0.90
anti-Tie2	3.1	Ang-F_1	27.2	82	3.0	0.96
anti-Tie2	3.1	Ang-F_2	27.4	85	3.1	0.99

Fig. 2 Analysis of multimericity, receptor-binding affinity and receptor-binding stoichiometry of angiopoietin variants. ***a***, Analysis of multimeric state of Angl and Ang2 variants by light scattering. ***b***, Ability of different Angl variants to compete for binding to immobilized Tie2-Fc. ***c***, Equilibrium binding of monomeric Ang-F_1 to Tie2-Fc. Tie2-Fc was first captured on the surface of a Biacore chip containing immobilized antibodies to Tie2-Fc. Varying concentrations of Ang-F_1 were then passed over the chip. Each trace represents the response to a particular concentration of Ang-F_1. Equilibrium values of the additional response unit (RU) observed correspond to ligand bound to receptor. ***d***, Plot of RU resulting from bound Ang-F_1 as a function of Ang-F_1 concentration. Each numbered point is derived from the correspondingly numbered trace in (*c*). The solid line results from fitting the points to a model of 1:1 binding stoichiometry. ***e***, Computed stoichiometries of Ang-F_1 and Ang-F_2 to Tie2. RU values derived from sensorgrams were used to calculate the mass of successively bound proteins, which were then converted to molar amounts. M_w is in kDa.

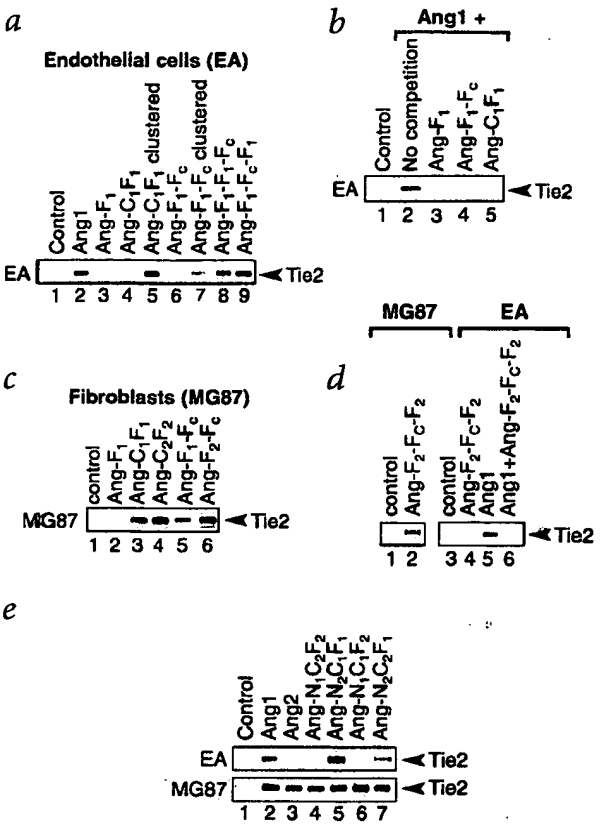

Fig. 3 Effects of angiopoietin multimericity and F domain chimeras on Tie2 activation. *a*, Monomeric and dimeric versions of Ang1 are unable to induce phosphorylation of Tie2 in EA.hy926 endothelial cells (EA) unless clustered, whereas tetrameric versions can stimulate phosphorylation without further clustering. *b*, Monomeric and dimeric versions of Ang1 inhibit Ang1-induced phosphorylation in endothelial cells. *c*, Dimeric but not monomeric versions of Ang1 and Ang2 are sufficient to induce phosphorylation of Tie2 in MG87 fibroblast cells. *d*, Engineered tetrameric version of Ang2 induces phosphorylation of Tie2 receptors in fibroblasts, but not in endothelial cells where it can block Ang1-mediated activation. *e*, Angiopoietin chimeras containing Ang1 but not Ang2 F domain can induce phosphorylation of endothelial Tie2 receptors. Ang-$N_1C_1F_2$ denotes a chimera consisting of the N and C domains of Ang1 fused to the F domain of Ang2; similar definitions apply for the other chimeras.

Angl (Fig. 3*b*, lane 5). In contrast, when ectopically expressed in fibroblasts, Tie2 could readily be activated by Ang-C_1F_1 (Fig. 3*c*, lane 3), indicating that activation of endogenous Tie2 receptors in endothelial cells is a more complex process (presumably because of co-expression of co-receptors or co-regulators) than activating ectopically expressed Tie2 receptors introduced into fibroblasts. Only the latter can be activated by ligand dimers.

One possible explanation for the finding that the dimeric Ang-C_1F_1 bound but could not activate endothelial Tie2 receptors was that native Angl is a higher oligomer than Ang-C_1F_1, perhaps because of further multimerization imposed by the N domain, and this higher degree of multimericity is absolutely required to activate endothelial Tie2 receptors. Consistent with such a possibility, clustering of Ang-C_1F_1 (by using an antibody directed against an N-terminal epitope engineered into this construct; Fig. 1*b*) activated endothelial Tie2 receptors (Fig. 3*a*, lane 5).

Multimeric structure of native Ang1

Native Angl had substantially stronger binding to Tie2 (~50–100-fold lower IC_{50} in competition experiments) than Ang-C_1F_1 (Fig. 2*b*), consistent with its having a higher avidity resulting from a higher multimerization state. To further compare the multimeric nature of Ang-C_1F_1 with native Angl, we used rotary shadowing transmission electron microscopy to directly visualize these molecules. The most frequent images obtained for Ang-C_1F_1 (Fig. 4*a*) were consistent with its dimeric state yielding 'mushroom-like' structures, consisting of an irregular bulbous 'cap' structure (presumably corresponding to the dimerized F domains) at one end of a 'stalk' structure (presumably corresponding to the double-stranded coiled-coil strands). Images for native Angl (Fig. 4*b*,*e*) support the hypothesis that native Angl involves further multimerization of this basic 'mushroom-like' building block, with the multimers coming together at a central point apparently corresponding to the location of the N regions (just upstream of the C domains). However, there was heterogeneity in the degree of multimerization because structures were noted that included two (a tetramer of F domains, Fig. 4*b*), three (a hexamer of F domains, Fig. 4*b*) or more (Fig. 4*e*) 'mushroom-like' building blocks.

These observations supported the notion that the N domains were responsible for the observed 'superclustering' of the basic dimeric building block. To test this prediction, mass spectroscopy of proteolytically generated peptides from native Angl was used to analyze the disulfide structure of the two cysteines, Cys21 and Cys34, in the N domains of Angl. A tryptic fragment was observed with a mass of 4,601.6 ± 1 Da, corresponding to a dimer of the tryptic peptide IQHGQC_{21}AYTFILPEHDGC_{34}R. This indicates that these two cysteines are involved in intermolecular links between distinct N domains (regardless of whether the dimer involves C_{21}–C_{34}/C_{21}–C_{34} or C_{21}–C_{21}/C_{34}–C_{34} linkages, which this analysis does not resolve). We were then able to construct a variety of models for the multimerization state of native Angl. In these models (Fig. 4*d*), disulfide linkages involving the N domains can form variable-sized ring structures, resulting in concatenation of increasing numbers of the Ang-C_1F_1 building blocks to form structures that are dimeric, tetrameric, hexameric and so on.

Minimal requirement for tetramerization

To confirm all aspects of this model, we genetically engineered precisely defined multimers of the F domain by completely substituting both the N and C domains with surrogate multimerization motifs. We first constructed a dimerized version of the F domain by fusing it at its C terminus to the Fc portion of human IgG1 (termed Ang-F_1-Fc; Fig. 1*b*). Fusion to the dimeric Fc dimerizes associated sequences, as was verified for Ang-F_1-Fc using light scattering (Fig. 2*a*). As predicted by the above model, this engineered dimeric Ang-F_1-Fc behaved just like Ang-C_1F_1 dimers. Ang-F_1-Fc bound to Tie2 about as strongly as Ang-C_1F_1 (Fig. 2*b*) and could activate ectopic Tie2 receptors introduced into fibroblasts (Fig. 3*c*, lane 5) but failed to stimulate endothelial Tie2 receptors (Fig. 3*a*, lane 6) and, likewise, inhibited their activation by Angl (Fig. 3*b*, lane 4). Moreover, further clustering of Ang-F_1-Fc using antibodies to Fc (Fig. 1*b*) allowed it to activate endothelial cell Tie2 receptors (Fig. 3*a*, lane 7), as was the case with clustering of Ang-C_1F_1 dimers. Thus, higher order clustering, induced with antibodies for either Ang-C_1F_1 or Ang-F_1-Fc and presumably brought about by the N domains in native Angl, seems to be essential for the ability of F domains to activate their endothelial Tie2 receptors.

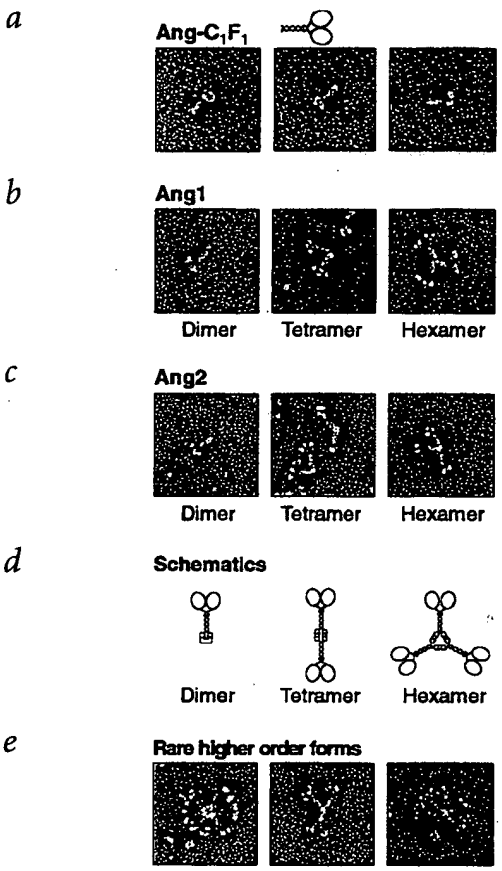

Fig. 4 Transmission electron microscopy visualization of angiopoietin oligomers. ***a***, Ang-C_1F_1 reveals characteristic 'mushroom-like' structure corresponding to dimers, as described in text. ***b***, Native Ang1 reveals variable-sized multimers of basic 'mushroom-like' structure. ***c***, Native Ang2 reveals multimers similar to those seen with native Ang1. ***d***, Schematic representations of angiopoietin structures. Cys21 and Cys34, both located in the N-terminal domain, contribute to the formation of variably sized ring structures, leading to the variable multimers. ***e***, Rare higher order forms.

To precisely determine the extent to which higher order clustering of F domains is required for Tie2 activation, we genetically engineered tetrameric versions of the F domain by placing two F domains in tandem, either followed or separated by a dimerizing Fc domain (termed Ang-F_1-F_1-Fc and Ang-F_1-Fc-F_1, respectively; Fig. 1*b*), with the latter verified to contain four fibrinogen-like domains by light scattering (Fig. 3*b*). These molecules with four F_1 domains were active on endothelial cell phosphorylation assays without the need for further clustering (Fig. 3*a*, lanes 8 and 9), demonstrating that precise tetramerization of Ang1 fibrinogen-like domains is sufficient to induce phosphorylation of Tie2 in cultured endothelial cells.

Stoichiometry of Ang1–Tie2 binding

The above findings are consistent with a model in which ectopic Tie2 receptors expressed in fibroblasts are similar to most receptor tyrosine kinases in that they are activated by simple dimerization induced by dimeric ligands. In contrast, native Tie2 receptors in endothelial cells require multimerization *via* minimally tetrameric ligands for their activation. To support the notion that a single F domain binds to a single receptor subunit and that ligand multimerization state reflects receptor multimerization state, we performed stoichiometric analysis using Biacore surface plasmon resonance (SPR). To avoid problems typically associated with chemical modification of either of the binding partners during the process of covalent immobilization on the sensor chip, we first immobilized antibodies directed against Tie2-Fc on the chip. Then, Tie2-Fc proteins were passed over the chip to allow noncovalent capture by the antibodies. The antibodies used (in independent experiments performed with two distinct antibodies) are nonblocking so that they do not interfere with the binding of Ang1 to Tie2 (see Methods). Therefore, the captured Tie2-Fc proteins should presumably have their Ang1 binding sites fully available. The amount of Tie2-Fc protein captured was determined from the response units (RU). Following this, increasing concentrations of monomeric Ang-F_1 were passed over the chip, and the amount bound at equilibrium was similarly determined from the additional RU observed (Fig. 2*c*). The amount of Ang-F_1 bound approached an asymptotic maximum as the concentration of free Ang-F_1 was increased, and this was taken as the amount of Ang-F_1 required to saturate the available binding sites on Tie2-Fc (Fig. 2*d*). After conversion of protein amounts to molar amounts, the amounts of captured Tie2-Fc and bound Ang-F_1 were compared. This analysis revealed that there was indeed approximately one monomer of fibrinogen-like domain for each monomer of Tie2 (Fig. 2*e*). Consistent with this, the shape of the binding curve conformed well to the prediction of a model of 1:1 binding stoichiometry, and the maximum binding predicted by the model was close to the observed maximum (Fig. 2*d*).

We cannot rule out the possibility that the binding stoichiometries in cellular contexts are not the same as in this *in vitro* context. Conceivably, endothelial Tie2 receptors bind Ang-F_1 dimers, thus requiring tetrameric forms to achieve receptor dimerization. However, taken together, the SPR and phosphorylation data are most plausibly explained by a binding stoichiometry of 1:1, a requirement for receptor dimerization in the non-endothelial context and a requirement for receptor tetramerization in the endothelial context.

Ang2 structure and function compared with Ang1

Although Ang2 shares ~55% amino acid identity with Ang1, Ang2 acts as a context-dependent agonist or antagonist, activating ectopic Tie2 receptors introduced into fibroblasts while antagonizing native Tie2 receptors on certain types of cultured endothelial cells[3]. If Ang2 were simply dimeric, these context-specific actions of Ang2 could easily be explained on the basis of the above observations with Ang1. Arguing against this possibility, rotary shadowing transmission electron microscopy revealed that Ang2 had essentially the same multimeric structure as had been seen for Ang1 — that is, Ang-C_2F_2 (the N-deleted version of Ang2) assumed a dimeric 'mushroom-like' structure analogous to that of Ang-C_1F_1, and native Ang2 consisted of variable multimers of these 'mushroom-like' structures (Fig. 4*c*). Because these results indicated that Ang1 and Ang2 had similar multimerization states, we next confirmed that the multimerization state of Ang2 was not responsible for its unique context-dependent agonistic/antagonistic capabilities by constructing a variant (Ang-F_2-Fc-F_2, analogous to Ang-F_1-Fc-F_1; Fig. 1*b*) containing precisely four Ang2 F domains (as confirmed by light scattering analysis; Fig. 2a). Ang-F_2-Fc-F_2 behaved as a context-dependent agonist/antagonist analogously to native Ang2 — that is, it activated fibroblast Tie2 receptors (Fig. 3*d*, lane 2) while failing to

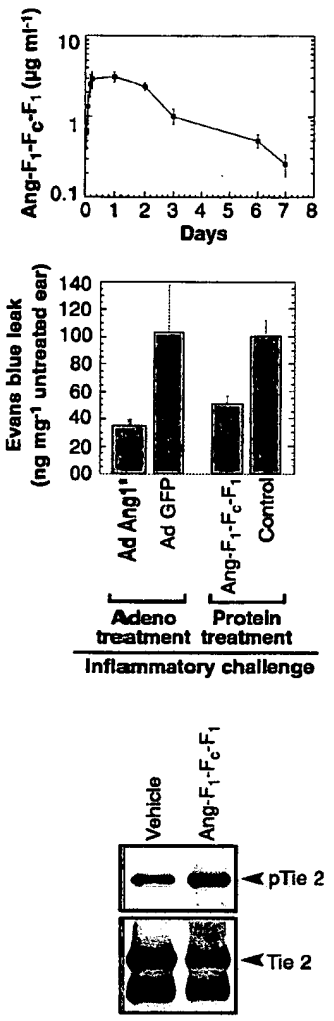

Fig. 5 Engineered tetramers of Ang1 are useful *in vivo* reagents. ***a***, Pharmacokinetic analysis of Ang-F_1-Fc-F_1 in mice, depicting circulating levels at indicated times after subcutaneous injection. ***b***, *In vivo* phosphorylation of Tie2 stimulated by subcutaneous injection of Ang-F_1-Fc-F_1, assayed 24 h after injection. Tie2 was immunoprecipitated from 1 mg of lung lysate and immunoblotted either with anti-phosphotyrosine to detect phosphorylated Tie2 (pTie2) or with an antibody against Tie2 to detect total receptor. ***c***, Systemic administration of Ang-F_1-Fc-F_1 protein causes resistance to mustard oil-induced vascular leakage comparable to resistance caused by adenoviral gene delivery. Plasma leakage of Evans blue was measured as described[19] in mice treated with Ang-F_1-Fc-F_1 protein or with adenoviral vectors expressing either Ang1* (Ad Ang1*) or green fluorescent protein (Ad GFP) as a control. Data are expressed as mean ± SEM (standard error of the mean) for 3 ears per group (n = 3).

activate endothelial Tie2 receptors (Fig. 3*d*, lane 4), and actually blocking Ang1-mediated activation of endothelial Tie2 receptors (Fig. 3*d*, lane 6). Biacore SPR experiments analogous to those performed with Ang1 showed that the stoichiometry of binding of Ang2 to Tie2 was also one F domain to one Tie2 monomer (Fig. 2*e*). Consistent with this, engineered dimeric forms of Ang2 could activate Tie2 in fibroblasts (Fig. 3*c*, lanes 4 and 6).

Taken together, these results strongly indicate that the unique context-dependent agonist/antagonist properties of Ang2 are not due to multimerization differences as compared with Ang1, and also indicate that the distinguishing features of Ang2 as compared with Ang1 are encoded within its F domain. To further confirm this possibility, chimeric molecules were made by exchanging either the N or F domains of Ang1 and Ang2. Chimeras containing the Ang1 F domain were agonists for endothelial Tie2 receptors (Fig. 3*e*, EA, lanes 5 and 7), whereas those containing the Ang2 F domain were context-dependent agonists or antagonists analogous to native Ang2 (Fig. 3*e*, lanes 4 and 6); as expected, all of these chimeras activated ectopic Tie2 receptors in fibroblasts (Fig. 3*e*, MG87).

Advantages of engineered Ang1 *in vivo*

Engineering precise multimers that maintain Tie2-activating ability has important practical consequences. The native Ang1 protein is heterogenous in size, difficult to produce and purify recombinantly, and cannot easily be detected upon injection into animals. Therefore, the *in vivo* actions of Ang1 have thus far been explored only using transgenic or gene therapy delivery schemes[17–19]. Because Ang1 delivery by these genetic schemes has suggested that Ang1 stabilizes vessels and prevents vascular leak in several inflammatory settings, a recombinant form of Ang1 that could be conveniently produced and provided *in vivo* could have significant therapeutic value. Ang-F_1-Fc-F_1, an engineered variant with four F domains, can apparently serve this purpose; it is easily produced and purified, displays excellent *in vivo* pharmacokinetics upon subcutaneous administration (Fig. 5*a*), rapidly causes *in vivo* phosphorylation of tissue Tie2 receptors (Fig. 5*b*) and successfully blocks vascular leak as effectively as genetically delivered Ang1 (Fig. 5*c*).

Conclusions

The angiopoietins have evolved unique ways in which to regulate their complex Tie2 receptor system. When the Tie2 receptor is ectopically expressed in fibroblasts, it seems to behave more like a conventional receptor tyrosine kinase and can be activated by simple dimeric ligands. In contrast, the native Tie2 receptor system in endothelial cells seems to be more complex, presumably because of the presence of co-receptors and/or co-regulators, and instead requires higher-order multimerization to be activated. To achieve such receptor multimerization, Ang1 uses a modular and multimeric structure unlike that of any other known growth factor, consisting of a C-terminal fibrinogen-like domain that is entirely responsible for receptor binding, a central coiled-coil domain that dimerizes these fibrinogen-like domains and a short N-terminal region that forms ring-like structures that supercluster dimers into variable-sized multimers. Genetic engineering of precise multimers revealed that Ang1 tetramers are the minimum size required for receptor activation, whereas dimers and monomers act as antagonists. Surprisingly, Ang2, which is a context-dependent agonist/antagonist of Tie2, displays a similar multimerization state to Ang1, and its unusual properties seem to be encoded within its fibrinogen-like domain. These emerging insights will certainly provide the basis for future studies into this fascinating but complex growth factor/receptor system.

Methods

Construction of variants. Ang1 and Ang2 variants and protein chimeras were constructed by PCR-directed mutagenesis. A trypsin signal sequence was used for those constructs that lacked their own signal sequence. The beginnings of the Ang1 coiled-coil and F domains were defined by the amino acid sequences FSSQKLQH and FRDCADVY, respectively. The corresponding sequences for Ang2 are DSVQRLQV and FRDCAEVF, respectively. Fc fusions were essentially as

described[13]. For Ang-F_1-F_1-Fc, the bridging sequence GPAP was inserted between the two tandem F domains. For Ang-F_1-Fc-F_1 and Ang-F_2-Fc-F_2, the bridging sequence GGGGSGAP was inserted at the end of the Fc before the second F domain. For Ang-C_1F_1, and Ang-C_2F_2, the FLAG epitope sequence, DYKDDDDK, was placed before the coiled-coil domain. Proteins were used either unpurified as COS cell supernatants or purified after transient expression in COS or CHO cells. All Fc-tagged proteins were purified by capture on protein A columns (Pharmacia). After loading, columns were washed extensively in 50 mM Tris and 500 mM NaCl, pH 7.5, eluted in Immunopure Gentle Elution Buffer (Pierce) and dialyzed extensively into Tris-buffered saline (TBS) containing 5% (v/v) glycerol. Aggregates were removed by size exclusion chromatography in the same buffer. Proteins that were not Fc tagged were purified by affinity chromatography. Human Tie2-Fc protein was first bound to Protein A Sepharose (2 mg Tie2-Fc per ml of resin), coupled with 5 mM Dimethyl pimelimidate•2 HCl (DMP; Pierce) in 25 mM K_2HPO_4, pH 8.75, and then blocked in the same buffer containing 50 mM glycine. Protein not coupled was removed with a 0.5 M glacial acetic acid wash before equilibration in phosphate-buffered saline (PBS), pH 6. Before affinity chromatography, cell supernatants were loaded onto cation exchange columns at pH 6.5 and eluted in 1 M NaCl, pH 8.5. The eluate was loaded onto the Tie2-Fc affinity column (after first subjecting the column to a mock elution) and then eluted in 3 M $MgCl_2$, followed by dialysis into TBS / 0.05% (w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), pH 7.5.

Light scattering experiments. Molecular weights (M_w) of Ang variants were determined using a Shimadzu SPD-10A UV detector, a MiniDAWN light scattering detector and a Shimadzu RID-10A differential refractive index detector, essentially as described[16].

Competition experiments. Competition experiments were performed with Ang1*, a modified form of Ang1 that is easier to produce and label in which the N domain of Ang1 is replaced by the N domain of Ang2 and the cysteine at residue 245 of Ang1 is replaced by a serine. Biotinylated Tie2-Fc (1 nM) was bound to Neutravidin-coated 96-well plates (Pierce) for 4 h at room temperature, followed by four washes with TBS / 0.1% (v/v) Tween-20 (TBST). Europium-labeled Ang1* (1 nM), combined with varying concentrations of competitor, was added to each well for 1 h at room temperature. Plates were washed four times with TBST, developed with 100 µl Enhance Solution (Wallac) and read using a Victor plate luminometer. Results are given as percent of maximum binding, where maximum binding is defined as the amount bound in the absence of competition. All measurements were done in triplicate. For Ang1* labeling, 1 mg of purified (as above) Ang1* in PBS / .05% (w/v) CHAPS, pH 8.5, was incubated overnight at room temperature with a 20-fold molar excess of DELFIA Eu-Labeling Reagent (Wallac) and then extensively dialyzed into TBS / .05% (w/v) CHAPS, pH 7.5. Yield was 2.1 Eu^{3+} per Ang1* monomer. The activity of labeled Ang1* in binding and phosphorylation assays was undiminished from that of unlabeled Ang1*. Tie2-Fc was biotinylated with 1-Biotinamido-44'-(Maleimidomethyl)Cyclohexane-Carboxamido (BMCC-biotin; Pierce) *via* a free cysteine located on the hinge region of the Fc tag.

Binding stoichiometry. The binding stoichiometries of Ang-F_1 and Ang-F_2 to Tie2-Fc were determined by SPR using a Biacore2000 instrument (Biacore) as described[20,21]. A CM5 sensor chip was first conjugated *via* primary amine chemistry with either a goat antibody specific to human IgG Fcγ (Jackson ImmunoResearch) or with a non-blocking monoclonal antibody to Tie2 (gift of K. Peters). Tie2-Fc was then injected over the antibody surface and the amount captured was recorded in RU. Varying concentrations of Ang-F_1 or Ang-F_2 were then injected over the Tie2-Fc, and the binding signals were recorded. The formula 1,000 RU = 1 ng protein mm^{-2} (set by the manufacturer) was used to convert binding signal from RU to mass (small corrections for glycosylation did not affect the results). All samples were in running buffer to minimize bulk effects. Subtracting the signal obtained from a reference surface not containing captured Tie2-Fc compensated for nonspecific effects. The M_w of Tie2-Fc was determined by light scattering, and that of Ang-F_1 and Ang-F_2 were determined by MALDI–TOFF. Binding data was fitted to a 1:1 model using BIAevaluation Version 3.2 (Biacore).

Phosphorylation assays. Tie2 receptor tyrosine phosphorylation was assayed in endothelial EA.hy926 cells[22] or in MG87 fibroblast cells stably transfected with Tie2 receptors[3] as described[1]. Ligand concentrations were in the range of 5–10 nM, except for Ang-F_1-Fc (20 nM) and Ang-F_1 (10 µM). For inhibition of Ang1-induced phosphorylation, 10 µM of monomeric (Ang-F_1) or 1 µM of dimeric (Ang-F_1-Fc or Ang-C_1F_1) ligands were used. For clustering experiments, cells were incubated for 5 min with the indicated protein, and clustering antibodies were added. For Ang-F_1-Fc, antibodies to human Fc were added at 10-fold molar excess for 5 min. For Ang-C_1F_1, anti-FLAG was added at 4-fold molar excess for 5 min, followed by the addition of anti-mouse at 10-fold excess for an additional 5 min. This additional clustering step was included to compensate for the fact that the antibody to FLAG could bind to both tags of a single Ang-C_1F_1 dimer without achieving higher order clustering.

Pharmacokinetic analysis. C57/bl6 male mice (25–30 g) were injected subcutaneously with 4 mg per kg of Ang-F_1-Fc-F_1 and tail bled at indicated times after injection (n = 9). All experiments were performed after approval by the Regeneron Institutional Animal Care and Use Committe (IACUC). Levels of circulating Ang-F_1-Fc-F_1 were measured by an ELISA assay based on capture with rat Tie2-Fc and detection with anti-human Fc.

Electron microscopy. A 20–200 µg ml^{-1} concentration of protein in TBS was carefully brought to 30–75% (v/v) glycerol and sprayed onto freshly cleaved mica (Pelco). Platinum/carbon was deposited under vacuum from an electron beam evaporator (Balzers) set 6° from horizontal with the stage at maximum rotation (~100 r.p.m.). The Pt layer was backed by a thin film of carbon applied normal to the rotating stage. Replicas were collected and examined with a JEOL 1200EX transmission electron microscope (JEOL USA).

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Competing interests statement

The authors declare competing financial interests: see the Nature Structural Biology website (*http://www.nature.com/naturestructuralbiology*) for details.

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corrigendum

Angiopoietins have distinct modular domains essential for receptor binding, dimerization and superclustering

Samuel Davis, Nick Papadopoulos, Thomas H. Aldrich, Peter C. Maisonpierre, Tammy Huang, Lubomir Kovac, April Xu, Raymond Leidich, Elzbieta Radziejewska, Ashique Rafique, Judah Goldberg, Vivek Jain, Kevin Bailey, Margaret Karow, Jim Fandl, Steven J. Samuelsson, Ella Ioffe, John S. Rudge, Thomas J. Daly, Czeslaw Radziejewski and George D. Yancopoulos

Nat. Struct. Biol. 10, 38–44 (2003).

Fig. 5 of this paper contains a mistake. The order of panels (*b*) and (*c*) should be reversed. For clarity, the entire figure is reprinted. We apologize for any inconvenience this may have caused.

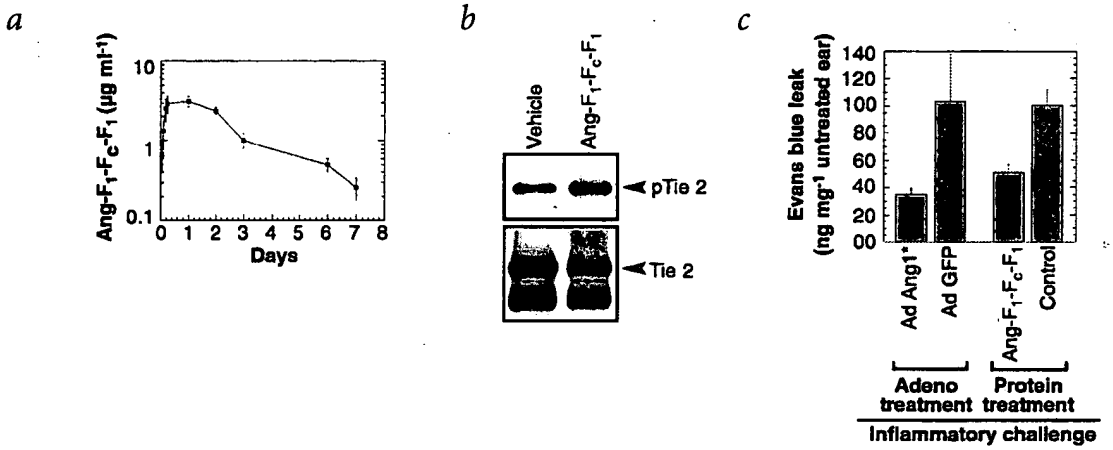

Fig. 5 Engineered tetramers of Ang1 are useful *in vivo* reagents. ***a***, Pharmacokinetic analysis of Ang-F_1-Fc-F_1 in mice, depicting circulating levels at indicated times after subcutaneous injection. ***b***, *In vivo* phosphorylation of Tie2 stimulated by subcutaneous injection of Ang-F_1-Fc-F_1, assayed 24 h after injection. Tie2 was immunoprecipitated from 1 mg of lung lysate and immunoblotted either with anti-phosphotyrosine to detect phosphorylated Tie2 (pTie2) or with an antibody against Tie2 to detect total receptor. ***c***, Systemic administration of Ang-F_1-Fc-F_1 protein causes resistance to mustard oil-induced vascular leakage comparable to resistance caused by adenoviral gene delivery. Plasma leakage of Evans blue was measured as described[19] in mice treated with Ang-F_1-Fc-F_1 protein or with adenoviral vectors expressing either Ang1* (Ad Ang1*) or green fluorescent protein (Ad GFP) as a control. Data are expressed as mean ± SEM (standard error of the mean) for 3 ears per group (n = 3).

erratum

Moving across membranes

Elaine A. Neale

Nat. Struct. Biol. 10, 2–3 (2003).

Reference 6 was inadvertently omitted from the reference list during the production process of this report. The missing reference is printed below. We apologize for any inconvenience this may have caused.

6. Ren, J. *et al. Science* **284**, 955–957 (1999).